



## Egg morphology update based on new chorionic data of *Potamanthus luteus* (Linnaeus), *Ephemera danica* Müller and *Oligoneuriella rhenana* (Imhoff) (Insecta, Ephemeroptera) obtained by scanning electron microscopy

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### Abstract

The chorionic patterns of Ephemeroptera eggs are very diverse and these have often been used for taxonomic and systematic purposes. In a great number of species, including *Potamanthus luteus*, *Ephemera danica* and *Oligoneuriella rhenana*, these egg features have been studied using light microscopy. However, current trends in egg morphology studies use scanning electron microscopy (SEM), so that the eggs of these species need to be re-described in order to establish morphological comparisons. The general chorionic features which have already been described in these three species are confirmed in our SEM study, although a more detailed description of both the architecture and arrangement of these can now be offered. In addition, this study has allowed us to note new morphological data, such as the chorionic reticulation in *P. luteus* and the complex extrachorion-adhesive layer in *E. danica*; classification of the lateral attachment structure in *P. luteus* and *O. rhenana* has been changed; and the variability of the polar cap observed in *P. luteus* underlines the care that must be taken when selecting chorionic structures for taxonomic purposes.

**Key words:** Ootaxonomy, egg attachment structures, micropyle, chorionic sculpturing, taxonomy, Potamanthidae, Oligoneuriidae, Ephemeridae

### Introduction

The eggshell surface in Ephemeroptera shows a great variability of morphological patterns, which has been used effectively many times to solve taxonomic problems or to establish systematic relationships (Belfiore *et al.* 1999; Degrange 1960; Domínguez & Cuezco 2002; Gaino *et al.* 1987, 1989, 1993; Mazzini & Gaino 1990; Klonowska-Olejniak 1997; Koss 1968; Koss & Edmunds 1974; Studemann & Landolt 1997; Thomas *et al.* 1999). The different morphological patterns are the result of a particular combination of chorionic structures in each species, especially as regards to shape, arrangement and distribution. Basically, chorionic structures can be categorized into three main classes, micropyles, attachment structures and chorionic sculpturing, which have been established mainly according to their physiological function. These types of chorionic structure, as well as the classification of each one given by Koss and Edmunds (1974), continue to be used as reference for morphological descriptions of Ephemeroptera egg. However, as this classification is based on light microscope observations, the increasing use of SEM as the normal technique for the description of egg morphology, is beginning to throw doubt on some of the old published data for many species and even the type assignation of many structures proposed in the new descriptions of eggs (Gaino & Mazzini 1988; Gaino & Bongiovanni 1992; Klonowska-Olejniak 2004; Mazzini & Gaino 1990; Ubero-Pascal 2004; Ubero-Pascal *et al.* 2005).

From a taxonomic point of view, the morphological features of the egg chorion are considered as useful as those of nymphs or imagoes in Ephemeroptera (Koss 1968; Koss & Edmunds 1974). For this reason, papers about egg morphology alone are becoming more numerous and it is common to find in papers on new species descriptions that include eggshell morphology as well as nymph and/or imago morphology (Alba 2000; Alba & El Alami 1999; Alba & Sowa 1987; Belfiore *et al.* 1999; Gaino & Bongiovanni 1993; Gaino & Puig 1996; Gaino *et al.* 1987; Jacobus & Sartori 2004; Kang & Yang 1994; Klonowska-Olejnik 2004; Klonowska-Olejnik & Jazdzewska 2003; Pescador & Edmunds 1994; Puig & Gaino 1996; Thomas *et al.* 1999; Towns & Peters 1979); unfortunately, many of these new descriptions are very superficial, despite being based on SEM analysis. On the other hand, the eggshells of many species are known only at light microscope level (Bengtsson 1913; Degrange 1956, 1960; Koss 1968; Koss & Edmunds 1974; Haybach 2003; Smith 1935), meaning that a comparison with eggs described by SEM cannot be recommended for taxonomic or systematic purposes. To resolve this problem, several authors have already undertaken a morphological re-description of these Ephemeroptera eggs for a specific taxon or group of species (Domínguez & Cuezco 2002; Kopelke & Mller-Liebenau 1981a, 1981b, 1982; Gaino *et al.* 1987, 1993; Malzacher 1982; Studemann & Landolt 1997; Studemann *et al.* 1995) based on SEM observations.

The eggs of *Potamanthus luteus* (Linnaeus, 1767), *Ephemerella danica* Mller, 1764 and *Oligoneuriella rhenana* (Imhoff, 1852) have been widely described at light microscope level by Degrange (1960), but have not yet been studied using SEM. In fact, few species of these genera and families have been studied using SEM (Balasubramanian *et al.* 1991; Kang & Yang 1994; Pescador & Edmunds 1994; Soldan & Thomas 1983). In this paper, therefore, we describe the fine morphology of the egg chorion of these three species in order to increase our knowledge of the morphology of the eggshell surface in Ephemeroptera eggs using SEM, confident that these data will be useful for future taxonomic and systematic studies.

#### Abbreviations used in figures

Ae = Aeropile; AL-Ex = Complex Adhesive-Extrachorion layer; C = Chorion; CM = Canal micropylar; CS = Chorion surface; D = Depressed central area; EV = Viteline Membrane; Ex = Extrachorion; FAL = Fibrous adhesive layer; FC = Fiber coils; Fl = Filament; TFC = Terminal fiber cluster; LA = Lateral attachment structure; MO = Micropylar opening; Mp = Micropyle; Ms = Reticula mesh; P = Protuberance; PB = Pedunculate base; PC = Polar cap; R = Reticulation; SG = Sperm guide; St = Reticula strand; TE = Thick edge; TK = Terminal knob

#### Material and methods

The three species, whose eggs have been studied, are widely distributed throughout the Palearctic-Occidental area, although all the specimens analyzed in this paper were collected in the Segura river basin (Spain) (Ubero-Pascal *et al.* 1998; Ubero-Pascal 2004): three nymphs of *P. luteus* from the Mundo river near Talave dam (525 m., 20/7/1994) and from the Segura river near La Graya village (615 m., 1/7/1993); two nymphs of *O. rhenana* from the Tus stream near Tus spa (1252 m., 24/6/1997); and two nymphs of *E. danica* from the Tus rapid (1300 m., 10/7/1994) and from the Madera stream near Segura de la Sierra village (1180 m., 10/7/1993) and, finally, one female imago of *E. danica* from Zumeta torrent near Santiago de la Espada village (1245 m., 9/7/1993). Female mature nymph (with black wingpads) were fixed in 4% formaldehyde and preserved in 70% ethanol, whereas imago were fixed and preserved in 70% ethanol. Eggs from the imago were studied only in the case of *E. danica*.

The eggs of each species were obtained directly from the abdomen of specimens and were processed for SEM study by the following procedure: cleared in a Branson 3510 ultrasound bath for 5 min; dehydrated in increasing concentrations of ethanol (80%, 90%, 95%) until absolute ethanol; dried using the critical point

method or air-dried after hexamethyldisilazane/tetramethylsilane treatment (see Ubero-Pascal *et al.*, 2005); stb mounted, sputter coated with gold-palladium and finally examined with Jeol JSM 6100 and Hitachi S3500N scanning electron microscopes. Forty to fifty eggs of each specimen studied were prepared for SEM analysis, verifying the morphological characteristics of the chorion in at least 10 of them.

For light microscopy analysis, the eggs were processed in order to obtain the cleared and stained effect produced by CMC-S mounting-medium that Koss (1968) used. As this mounting-medium is no longer commercially available, the eggs were stained with neutral red and mounted on slides with “hoyer” mounting-medium. Eggs on slides were analyzed three or four weeks after preparation with a Leica DMRBB microscope.

The terminology and classification of the chorion structures proposed by Koss and Edmunds (1974) were followed in the descriptions of egg morphology.

### ***Potamanthus luteus***

#### Description of egg morphology

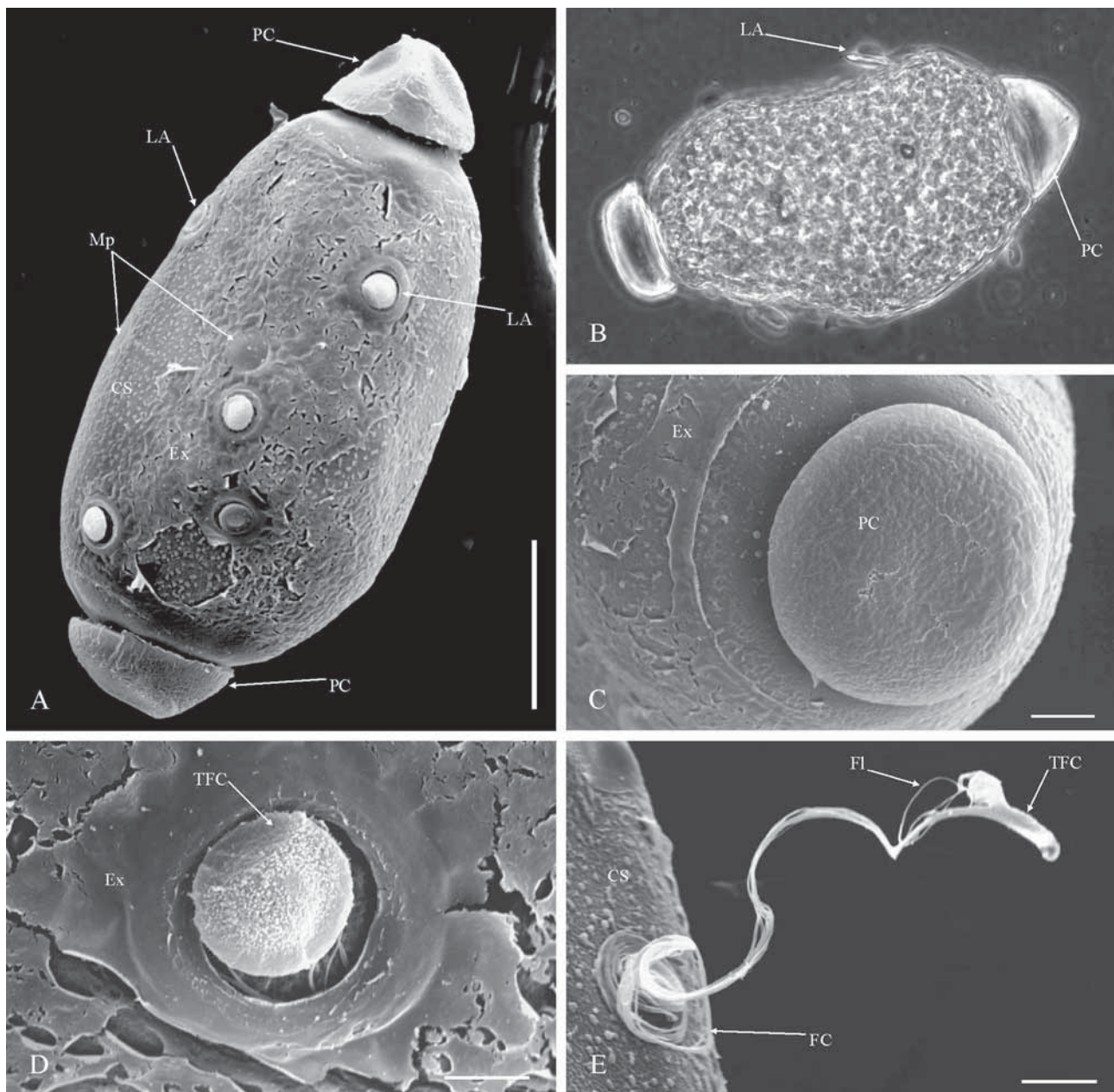
**General features:** 170–179  $\mu\text{m}$  length and 91–109  $\mu\text{m}$  width. Egg oval-shape with round poles and a polar cap on each one, numerous lateral attachment structures on both subpolar areas, and some micropyles on equatorial area (Fig. 1A). These characteristics are always observable even though the egg is covered by an extrachorion, this layer sometimes hiding other chorionic structures (Fig. 2B)

**Attachment structures:** These types of chorionic structure are fibrous and, due to both the organization of the filaments and their relative position on the eggs, may be identified as a polar cap (epithemata) or lateral attachment structure. The polar cap is formed by countless filaments, which are slightly thickened apically, arranged in parallel and tightly packed beneath a thin layer (the extrachorion) that covers them (Fig. 1A, C); for this last reason, the filaments are not visible by SEM unless the extrachorion is removed. The organization of this chorionic structure corresponds to “type I”. Generally, the polar caps have a conical form (Fig. 1A) and variable dimensions (52–45  $\mu\text{m}$  in length and 33–20  $\mu\text{m}$  in width), although they may be flattened in some eggs (Fig. 1C). The eggs can show polar caps of the same or different forms (Fig. 1A, B). The polar caps cover only the most apical zone of the egg polar area and, depending on its form (conical or flattened), they can change the apparent egg-shape from oval-like to elliptical-like or barrel-like (with poles truncated).

The lateral attachment structure is a coiled fiber, finishing distally in a round and flattened fibrous expansion (Fig. 1E) which, according to the classification of attachment structures proposed by Koss & Edmunds (1974), could correspond to the type “fiber-coils with terminal fiber clusters”; although, the shape of this structure under the light microscope (Figs. 1B and 2A) or as seen by low increases in SEM (Fig. 1A) can easily be confused with the type “knob-terminated coiled threads” (KCT). The fiber is formed by numerous filaments of different thickness loosely-arranged (Fig. 1E), coiling directly over the egg chorion and forming a roll with a central hollow, in which the terminal expansion is arranged. Terminal expansion is formed by numerous microfilaments, with the appearance of a velvet pad and a diameter of 8.9–12  $\mu\text{m}$ . Extrachorion covers the fiber coil but not the terminal expansion (Fig. 1D), thus maintaining the attachment structure coiled, when this thin layer is lacking the fiber is uncoiled (Fig. 1E). The lateral attachment structures are distributed in zig-zag round the egg, with a minimum number of 4 units in each subpolar area (Figs. 1A and 2B). Some of them may appear in the equatorial area but very rarely.

**Chorionic sculpturing:** Extrachorion usually hides the chorionic surface (Fig. 2B), but when this layer is cleared, two types of chorionic sculpturing can be differentiated: tubercle-like protuberances and a slight irregular reticulation (Fig. 2C). The protuberances are pedunculate (Fig. 2C) and variable in size (0.5–1.7  $\mu\text{m}$  height and 0.9  $\mu\text{m}$  width); sometimes, several units are fused. Reticulation is only clearly appreciable by SEM at 1,000X, and can be considered an irregular net of small mesh, whose mesh-units are slight depressions that vary considerably in both form and size (0.38–0.93  $\mu\text{m}$  wide) (Fig. 2C).

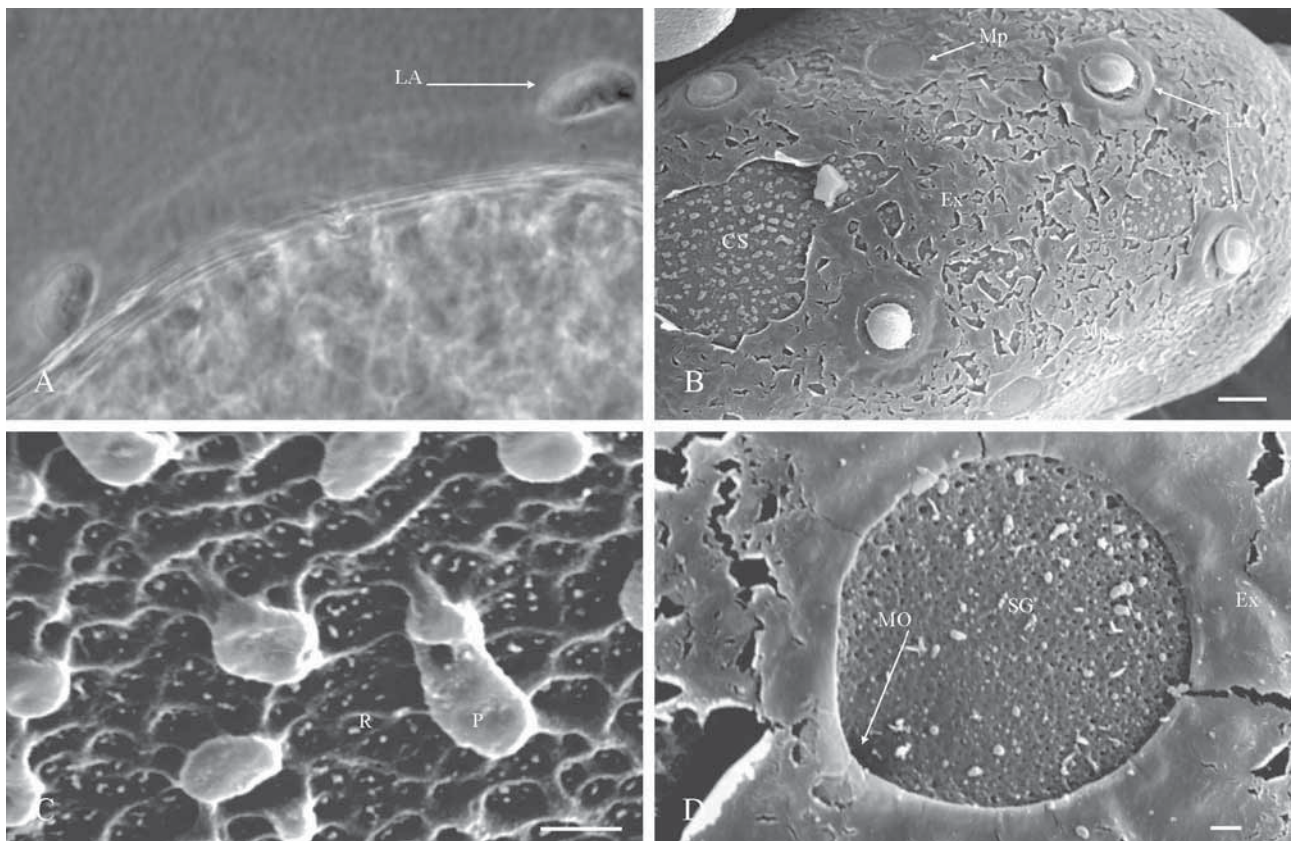




**FIGURE 1.** Egg morphology of *Potamanthus luteus*: A, General shape of egg with conical polar caps (scale bar = 50  $\mu$ m). B, General shape of eggs by light microscope, showing flat and conical polar caps and many lateral attachment structures (40x). C, Flat polar cap form (scale bar = 10  $\mu$ m). D, Fiber-coils with terminal fibers cluster coiled (scale bar = 5  $\mu$ m). E, Fiber-coils with terminal fibers cluster uncoiled (scale bar = 10  $\mu$ m).

**Micropyles:** The sperm guide and the micropylar opening are the only parts of micropyle observable by SEM (Fig. 2D), since the micropylar canal is completely intrachorionic; however, observation of the micropylar canal with the light microscope shows that this micropyle is of the tagenoform-type. The sperm guide has a circular form (10–13.1  $\mu$ m diameter) and is easily distinguishable because it is delimited perfectly by extra-chorion and the chorion lacks the chorionic sculpturing pattern indicated previously in that zone (Fig. 2D). Therefore, the sperm guide could be assigned to the chorionic-suprachorionic type. The micropylar opening is a circular-shaped orifice, infrachorionic, and almost perpendicular to the chorion surface. This orifice constitutes the beginning of a micropylar canal, whose cross-section decreases as it enters the chorion. The egg presents several micropyles. We have observed three units as least, although there could well be more, and they

are distributed linearly around the egg equatorial area (Fig. 2D), although sometimes some of them may be displaced towards the subpolar areas.



**FIGURE 2.** Egg morphology of *Potamanthus luteus*: A, General shape of fiber coils with terminal fibers cluster by light microscope (100x). B, Extrachorion and arrangement of lateral attachment structures and micropyles (scale bar = 10 µm). C, Chorionic sculpturing in detail (scale bar = 1 µm). D, Micropyle: sperm guide outlined by the extrachorion (scale bar = 1 µm)

#### Partial discussion

The most complete description of egg morphology of *P. luteus* is that offered by Degrange (1960), although, as this author acknowledges, some data were already known from the XIX century. Isolated egg data for this species can also be consulted in Degrange (1956) and Haybach (2003). The morphological features described by Degrange for the two types of attachment structures, the micropyle and the chorionic sculpturing, as well as the position of these structures on the egg chorion, agree perfectly with our SEM observations. Nevertheless, other chorionic features, such as irregular reticulation and extrachorion, and the fine detailed arrangement of the lateral attachment structure, are described for the first time in this paper. Probably, Degrange could not observe the irregular reticulation of the chorion because this feature would be beyond the optical resolution of the light microscope. On the other hand, the technique used by Degrange to prepare the eggs for study could be related with the non-observation of extrachorion, since this layer is transparent and disappears when unfixed eggs are put in water; however, in other species he described a similar film envelope on eggs preserved in ethanol.

Based mainly on the morphological descriptions of eggs provided by Degrange (1960) and Koss (1968) for some species of *Potamanthus*, Koss and Edmunds (1974) established three morphological features that characterized Potamanthidae eggs: “polar caps type I”, tuberculate-like protuberances and lateral attachment structures of the type “knob-terminated coiled threads”. We are inclined to maintain this classification, except



in the case of the lateral attachment structure, based on SEM observations which clearly show ultrastructural differences with respect to a typical thread (see KCT's on Heptageniidae eggs, for example in Gaino & Reborá 2003: figure 6; Ubero-Pascal 2004: figure 6.27D). According to Koss and Edmunds (1974), a thread is a tight spiraling of fibers forming a polyfilamentous structure, but the ultrastructure of the lateral attachment structure on *P. luteus* is nearer to a loose collection of monofilaments or fibers. These two types of fibrous structure could be easily confused at light microscope level when their uncoiled shape is similar and, indeed, this is the case with *P. luteus*. But by SEM, the differences are clearer although the structure still looks uncoiled; therefore, when analyzing the photographs of eggs of *P. (Potamanthodes) idiocercus* Bae & McCafferty, 1991 (Kang & Yang 1994), we think that this egg has “fiber-coils with terminal fiber clusters” as lateral attachment structure, and not KCT, as has been described. Probably, the Potamanthidae eggs have this type of lateral attachment structure as a characteristic, but we must be cautious in this respect and examine egg morphology by SEM in more species.

Variability in the form of polar caps in eggs from the same specimen have already been described in Ephemeroptera, but until now we have no information available as to whether this also occurs in *P. luteus* eggs. Polar cap variability was detected in eggs of *Serratella ignita* (Poda, 1761), being related the polar cap shape with the egg position in the oviduct (Bengtsson 1913; Degrange 1960) or in the clump of eggs hanging from the end of the imago abdomen before laying (Gaino & Bongiovanni 1992). Unfortunately, we found no evidence that allowed us to relate the polar cap variation in eggs of *P. luteus* with anything. According to Gaino and Bongiovanni (1992), the different forms of polar caps show clear ultrastructural differences and could be the result of changes in the secretory activity of follicular cells during oogenesis. Probably, the variability of polar caps in *P. luteus* is produced by the same phenomenon, but a deeper study is necessary to confirm this.

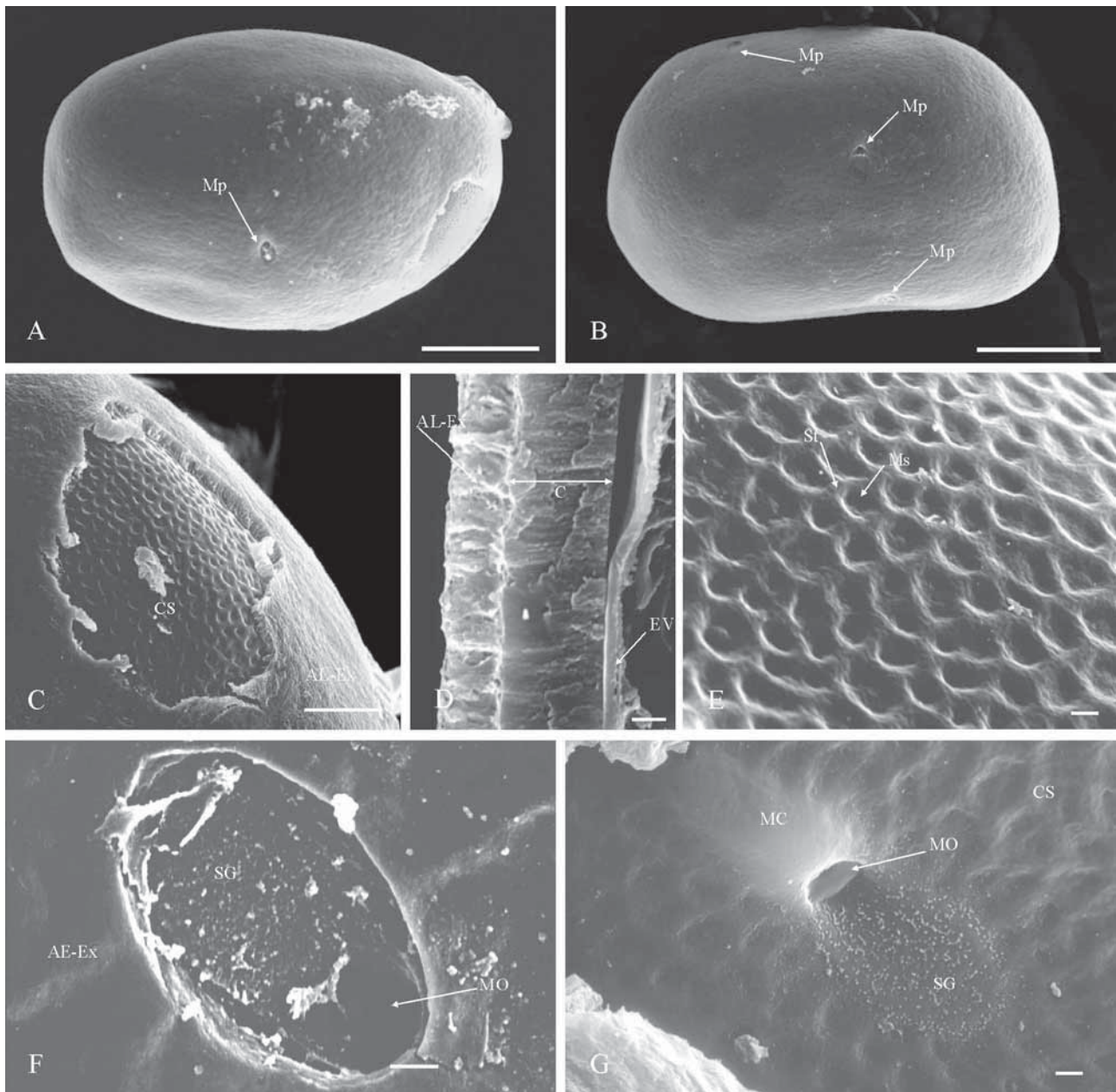
Koss and Edmunds (1974) suggested that chorionic sculpturing is the main structure useful for species differentiation in *Potamanthus*, since they found size variations in the tubercle-like protuberances of the species studied. We agree with this proposition, but not only on the basis of tubercle size variations, but also because this type of chorionic structure can be accompanied by others, such as the net-like of small mesh that we described in *P. luteus*. Therefore, chorionic sculpturing in *Potamanthus* eggs may be more complex, as well as species-specific, than a simple variation in tubercle size. It was probably the limited resolution of the optical microscope or presence of extrachorion that led protuberances to have been described as merely ornamentation before our study. In this respect, we emphasize that extrachorion may mask the true ornamentation of the chorion surface in SEM studies of egg morphology, which is probably what happened to Kang and Yang (1994).

According to Degrange (1960), *P. luteus* identification by reference to egg morphology is no problem in the Western Palearctic Region because the egg pattern is singular and unmistakable; it is also the only species of Potamanthidae in this area. From a global point of view, SEM morphological descriptions of eggs in a greater number of species of *Potamanthus* would be needed to establish comparisons and relationships, since those that exist are few and superficial.

### *Ephemera danica*

#### Description of Egg Morphology

**General features:** Oval-like egg (Fig. 3A), although sometimes quadrangular (Fig. 3B) or with irregular form. Very simply egg shape, with smooth surface due to the “complex adhesive-extrachorion layer” which completely surrounds the egg (Fig. 3A, B). Several micropyles have been observed in the equatorial area, frequently some may be displaced towards the subpolar area (Fig. 3A, B). Egg-size between 175–209 µm length and 114–128 µm width.



**FIGURE 3.** Egg morphology of *Ephemera danica*: A, General shape of egg oval-like. B, General shape of egg quadrangular-like (B) (scale bar = 50 µm). C, Detail of egg surface without complex extrachorion-adhesive layer showing the chorionic sculpturing (scale bar = 10 µm). D, Detail of thickness and complexity of layer forming the eggshell (scale bar = 1 µm). E, Chorionic sculpturing in detail (scale bar = 1 µm). F, Trace of sperm guide of micropyle in the complex extrachorion-adhesive layer (scale bar = 1 µm). G, Trace of sperm guide and proximal part of micropylar canal on chorion surface (scale bar = 1 µm).

**Attachment structures:** An adhesive layer is the only attachment structure that the egg possesses. This layer covers the whole egg, hiding the chorion surface completely, except at the sperm guide of each micropyle (Fig. 3B). The adhesive layer is a compact material of granular or undifferentiated appearance (Fig. 3C), whose thickness is approximately half that of the chorion layer (Fig. 3D). The extrachorion is a very slight film covering the adhesive layer and its differentiation is very difficult, even in a cross-section of the eggshell (Fig. 3D), probably because it and the adhesive layer are strongly adhered and form a single unit. We therefore prefer to call this attachment structure as a “complex adhesive-extrachorion layer”.

**Chorionic sculpturing:** The chorionic features are only observed by SEM when the adhesive layer covering its surface has been removed (Fig. 3A, C). Chorionic sculpturing consists of small depressions of circular-shape (1.3–2.9  $\mu\text{m}$  diameter), whose distribution and arrangement extend regularly the whole chorion surface and could be related to a small meshed reticulation pattern (Fig. 3E). The mesh of net-like sculpturing consists of small depressions and a polygonal reticulum ridge-like strand with hexagonal, pentagonal or irregularly shaped units (Fig. 3E)

**Micropyles:** The micropyle are “tagenoform-type”, with an oval shaped sperm guide (Fig. 3F), sometimes almost elliptical, followed by a long intrachorionic tube, the micropylar canal. If the complex extrachorion-adhesive layer is present, the sperm guide is the only part of the micropyle that can be differentiated by SEM, because it is perfectly delimited by this attachment structure (Fig. 3F). When the complex layer is removed, the sperm guide can be observed since this area does not present the typical chorionic sculpturing (Fig. 3G); therefore, the sperm guide is a “suprachorionic-chorionic” type. In these circumstances, the proximal zone of the micropylar canal can also be observed (Fig. 3G). Sperm guide size is 7.4–8.5  $\mu\text{m}$  length x 5  $\mu\text{m}$  width.

The number of micropyles appearing in an egg is variable and we have observed a maximum of three (Fig. 3B). These are arranged in the equatorial area of the egg, more or less aligned in a cross-sectional direction, and separated by a distance close to half the total width of the egg (Fig. 3B). Sometimes, one of these micropyles can be displaced to the subpolar area (Fig. 3B)

#### Partial discussion

The egg morphology of *E. danica* was described in detail by Degrange (1960), although partial descriptions and isolated data can also be found in Bengtsson (1913), Degrange (1956), Koss and Edmunds (1974) and Haybach (2003). The characteristic morphology of eggs given by Degrange (1960), concerning attachment structures and chorionic sculpturing, is re-described in the study, although our observations of micropyle morphology are closer to the description given by Koss and Edmunds (1974).

We regard the attachment structure in the eggs of *E. danica* as a complex formed by an adhesive layer and the extrachorion. The adhesive layer is closer to the chorion surface and, in agreement with Degrange (1960) and Koss and Edmunds (1974), it has a granular or amorphous appearance, but never fibrous. The extrachorion is a delicate film covering the adhesive layer surface, almost imperceptible in cross-section, but the smooth appearance of the egg surface is due to its presence. The close relationship between the adhesive layer and the extrachorion is not limited to form a structural complex, but also extends to a physiological function. Probably, the presence of extrachorion would explain the different physiological response of the adhesive layer observed by Degrange (1960) when the eggs were submerged in physiological liquid or merely in water.

This “complex extrachorion-adhesive layer” is a chorionic structure common to all *Ephemera* eggs, as well as to all Ephemeridae eggs (Degrange 1960; Koss 1968; Koss & Edmunds 1974). Its presence should be taken into account in SEM studies of egg morphology, because it can hide the chorionic sculpturing or prevent the correct observation of the micropyle. Chorionic sculpturing in *Ephemera* eggs is very common and its specific features have been described in many species, since this chorionic characteristic can be differentiated easily by light microscopy (Degrange, 1960; Koss, 1968; Koss & Edmunds, 1974). For these reasons, we think it necessary to treat with caution SEM descriptions of egg morphology that do not consider the presence of an adhesive layer, such as in Balasubramanian et al. (1991) and Kang and Yang (1994). Some eggs, which partially lost their attachment structure during the preparation procedure for SEM analysis, confirmed the observation of Degrange (1960), who mentioned that *E. danica* chorion presents a net-like sculpturing of small mesh, although reticulation can be polygonous irregular or not.

In agreement with the observations of Koss and Edmunds (1974), the sperm guide is present in *E. danica* eggs and so is the micropyle, the “tagenoform-type”, but not the “linear-type” or “type 4” according to Degrange (1956, 1960). The sperm guide leaves traces on both the chorion and “complex extrachorion-adhesive layer” (“suprachorionic-chorionic” type) but, while the trace in the attachment structure is very conspicu-



ous (it is a well delimited hole), on the chorion surface it is poorly defined since it is only differentiable due to the absence of typical chorionic sculpturing. Probably, the sperm guide was less neatly defined in the eggs studied by Degrange (1960) (fresh-eggs and each enveloped by a thick transparent adhesive layer) and he could only observe it when the eggs were stained by aniline blue, differentiating an oblong hole in the adhesive layer just at the micropyle opening. Overlapping of both sperm guide traces could be the reason that Degrange (1960) did not observe the sperm guide trace on the chorion surface when the eggs were stained. On the other hand, the micropylar canal features observed by us are nearer the characteristics described by Degrange (1960) than by Koss and Edmunds (1974). The proximal part of the micropylar canal is slightly raised with respect to the chorion surface, but the absence of chorionic sculpturing in this area means that this feature seems more pronounced. In any case, the proximal part of the micropylar canal does not project above the chorion as it does *Caenis* eggs (Malzacher 1982).

From a taxonomic point of view, a non-fibrous adhesive layer as the only attachment structure and a "tageniform" micropyle seem to be common features of Ephemeridae eggs, although *Ephemera* eggs could be characterized because the adhesive layer lacks any type of sculpturing. According to Koss and Edmunds (1974), chorionic sculpturing seems to be the only egg characteristic which would allow the identification of species of *Ephemera*, since its variability could be species-specific. However, although Degrange (1960) found differences in the chorionic sculpturing of eggs from the three species of *Ephemera* studied, he did not consider them to be of taxonomic use; in fact, his proposed key for species differentiation based on egg morphology has only one entry covering these three species of *Ephemera*. Without doubt, a SEM study of the eggs of several *Ephemera* species would help decide whether chorionic sculpturing is useful for taxonomic purposes. In the Segura river basin, *E. danica* occurs with other *Ephemera* species (*E. glaucops* Pictet, 1843 and *E. lineata* Eaton, 1870) (Ubero-Pascal *et al.* 1998), but, unfortunately, we did not find specimens with eggs of these species. Hopefully, specimens with eggs will be found so that they can be compared with the *E. danica* data described in this paper.

### ***Oligoneuriella rhenana***

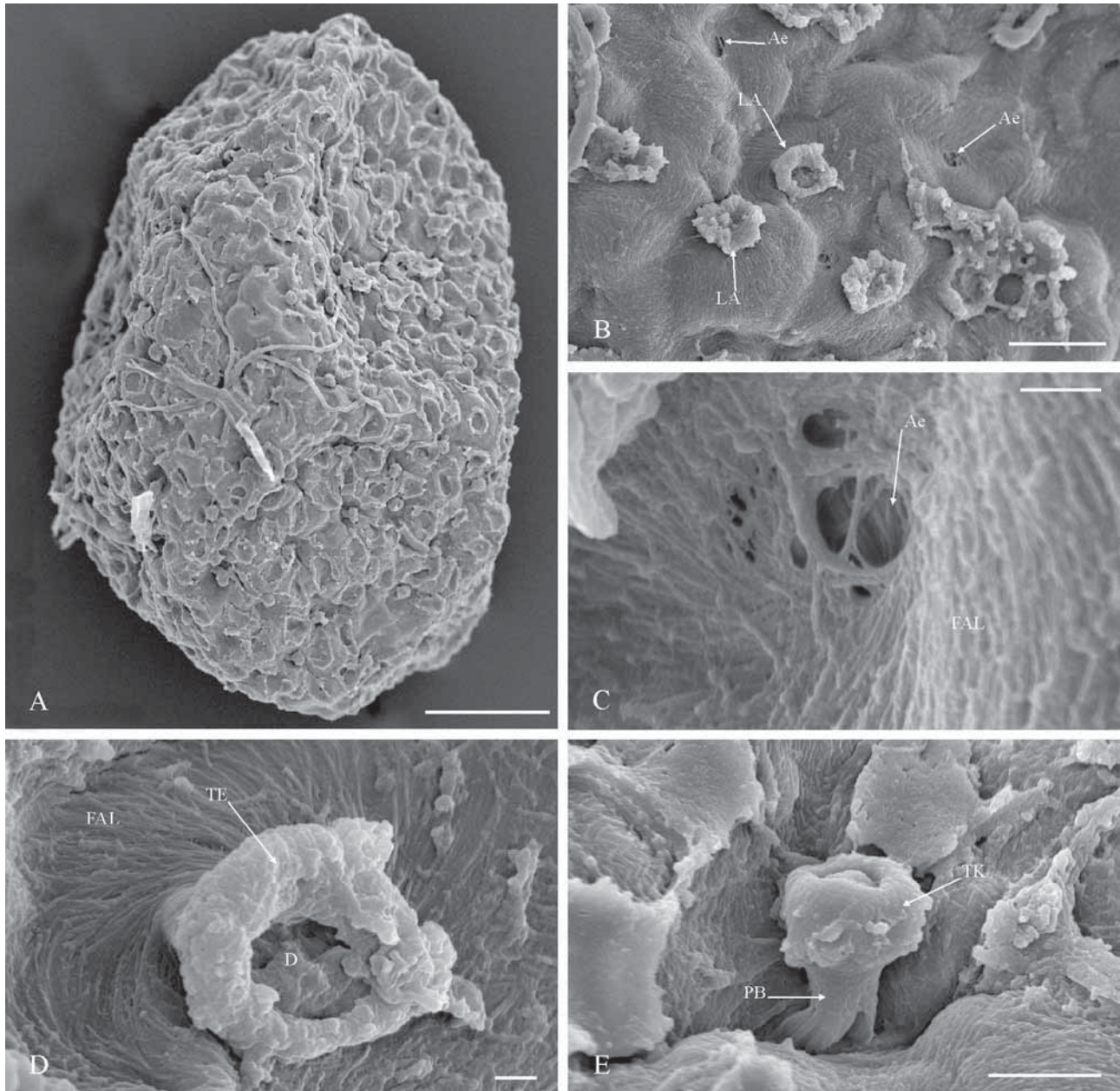
#### Description of egg morphology

**General features:** Oval-like eggs with slightly pointed poles, morphologically characterized by a wavy chorion surface, although apparently smooth at low magnifications, with many attachment structures which are distributed uniformly (Fig. 4A, B). The eggs of this species are big and their dimensions vary between 264–297  $\mu\text{m}$  in length and 161–208  $\mu\text{m}$  in width

**Attachment structures and chorionic sculpturing:** These two basic characteristics of eggs must be analyzed jointly in this species, because all the chorionic structures observed externally are related to an attachment function, but at the same time constitute the egg sculpturing, since they cover the egg completely. The chorion surface is a fibrous adhesive layer strongly adhering to the rest of the chorion, whose fibers may be distinguished clearly by SEM from 2000x magnifications. The fibers are formed of short and fine filaments (0.11  $\mu\text{m}$  diameter) arranged in parallel forming bundles (Fig. 4C, D). In addition, these bundles of filaments may be related with each other following the same direction or intercrossing (Fig. 4 B, C), causing different ultrastructural levels of organization in the thickness of the fibrous layer. The fiber arrangement also causes the appearance of different types of external structure, including small orifices ( $\pm 2$   $\mu\text{m}$  of diameter) and fibrous projections (Fig. 4C, D). The egg surface presents many such orifices randomly distributed, and often can be observed in them, although with difficulty, that the fibers can be seen to be organized into several layers (Fig. 4C). These orifices are probably related into aeropyles, although a detailed study would be necessary to confirm this.

In certain points of the fibrous layer, one or more bundles of filaments rises from the layer surface, turning spirally to produce fibrous projections, that could be classified as “fiber-coils”, of which two types can be distinguished, without a terminal fiber clusters and with a terminal knob. “Fiber-coils without terminal fiber clusters” correspond to a round structure of 6.5  $\mu\text{m}$  diameter and sucker-shape, in which the filaments form a thick edge that delimits a depressed central area (Fig. 4D). On the other hand, “fiber-coils with terminal knob” correspond to a pedunculate structure and mushroom-shape (Fig. 4E), approximately 7.3  $\mu\text{m}$  height and 5.8  $\mu\text{m}$  diameter in the umbrella-shaped part. This last structure has a more compact appearance than the previous one and its fibrous nature is only observable in the peduncle base, for which reason it has been classified as “fibrous attachment structure”.

**Micropyles:** These basic chorionic structures were not found in the eggs of this species.



**FIGURE 4.** Egg morphology of *Oligoneuriella rhenana*: A, General shape of egg (scale bar = 50  $\mu\text{m}$ ). B, Detail of adhesive layer surface and lateral attachment structures (scale bar = 10  $\mu\text{m}$ ). C, Detailed of orifice in adhesive layer (scale bar = 1  $\mu\text{m}$ ). D, Fiber coils without terminal fibers cluster in detail (scale bar = 1  $\mu\text{m}$ ). E, Fiber-coils with terminal knob in detail (scale bar = 5  $\mu\text{m}$ ).

## Partial discussion

The main morphological aspects of *O. rhenana* egg were dealt with in depth by Degrange (1960) and later, briefly, by Haybach (2003). Our SEM observations confirm the fibrous nature of the attachment structures (layer surrounding the chorion and lateral projection), that has already been described by Degrange (1960) in this species, which is a very common feature in Oligoneuriidae eggs (Koss & Edmunds 1974). The morphology and arrangement of the fibers of the fibrous layer of *O. rhenana* and *Siphonurus lacustris* (Eaton, 1870) eggs were very similar according to Degrange (1960), but SEM investigations suggest quite the opposite. The fibers of *O. rhenana* are short and compact bundles, formed by tiny inconspicuous filaments, which may be arranged in parallel or intercrossed, whereas the fibers of *S. lacustris* are very long, relatively thick, and very conspicuous; they are loosely arranged in different directions (Gaino & Rebora 2001). The ultrastructural organization between the fibrous attachment layer and the rest of the chorionic layers is still unknown in *O. rhenana*, and a study of the eggshell using TEM is necessary.

The lateral attachment structures in *O. rhenana* eggs have been classified as fiber-coils with terminal fiber clusters, although two forms have been distinguished, one apparently evolved from the other (Degrange 1960; Koss & Edmunds 1974). In our SEM study, two forms of fiber-coils were also differentiated; however, in relation with the forms already described, morphological differences are evident but we found no clear indication concerning which one evolves from the other. In fact, if we simply look at the organization of the distal end, we can differentiate two types of fiber-coils: those with a terminal knob and those without a terminal fiber cluster. Our fiber-coils with a terminal knob only have a lateral attachment structure which arises from the eggshell surface as a projection, and which is only a part of the attachment structures included in the type described by Koss and Edmunds (1974). The fiber-coils without terminal fiber clusters involve the remaining lateral attachment structures, which appear to terminate within themselves, forming a sucker-shaped structure. A similar structure to this last one has already been described by Koss and Edmunds (1974) for Oligoneuriidae eggs, but not in *O. rhenana* eggs. In addition, having obtained SEM evidences of the morphology of fiber-coils in eggs of *S. lacustris* (Gaino & Rebora 2001) and in *O. rhenana*, we were able to verify that these are clearly different, both in morphology and in arrangement unlike that indicated by Koss and Edmunds (1974), in which *S. lacustris* fiber-coils are really of the type with terminal fiber clusters.

The extrachorion could not be determined in the eggs of *O. rhenana* studied, although this cover is probably present and could be the thin layer that Degrange (1960) described enveloping the attachment structures externally which coagulated in eggs preserved with ethanol. We cannot explain why we did not observe this layer in *O. rhenana*, having studied eggs preserved in ethanol and obtained from nymphs, even though the extrachorion was observed in the eggs of other species preserved in ethanol (Ubero-Pascal *et al.* 2005); according to Koss and Edmunds (1974), eggs from nymphs display a thicker extrachorion. Neither did we observe a micropyle in eggs of *O. rhenana*, although this chorionic structure is “tagenoform-type” and one or two units appear near one of the polar areas (Degrange 1960). Probably, as already described in other species displaying a fibrous attachment layer (Gaino & Rebora 2001), the micropyle can only be observed by SEM when this layer has been removed.

The fibrous adhesive layer and “fiber-coils” as lateral attachment structures seem to be common characteristics of the morphological patterns of Oligoneuriidae eggs. From a taxonomic point of view, the density and distribution of the lateral attachment structures around the egg could help differentiate species, although their use in genera taxonomy is not clear from light microscopy (Koss & Edmunds 1974). Descriptions of the chorion morphology in Oligoneuriidae using SEM are scarce, very concise or merely represented by a photograph (Pescador & Edmunds 1994; Soldan & Thomas 1983), while our description of *O. rhenana* eggs demonstrates that SEM can show new details of the chorionic structure which could be taxonomically useful, for example for differentiating from *Oligoneuriopsis skounate* Dakki & Giudicelli, 1980. This species occurs with *O. rhenana* in the Segura river basin and, although we only have a general photograph of its egg (Soldan & Thomas 1983), both species could be differentiated by the “fiber-coils”. *O. skounate* has eggs with “fiber-



coils a with terminal fiber clusters", very similar in morphology to the egg of *S. lacustris*, while the two types of "fiber-coils" of *O. rhenana* egg are clearly different.

## Conclusions (General remarks)

The chorionic features that define the morphological pattern of eggs in *P. luteus*, *E. danica* and *O. rhenana*, which were reported mainly by Degrange (1960) using light microscopy, have been confirmed in our study. However, the use of SEM allowed us to describe in more detail the fine morphology of these structures. In this way, new chorion structures previously undescribed, such as the reticulation in *P. luteus*, have been observed, and we have also been able to verify that the ultrastructure or arrangement in other chorionic structures do not fit with published descriptions, for instance the lateral attachment structures in *P. luteus* and *O. rhenana*. The presence of orifices in the surface of the attachment layer in *O. rhenana* is probably related with aeropyles, since similar structures have already been observed in the eggs of other species of Ephemeroptera (Mazzini & Gaino 1985; Ubero-Pascal 2004; Ubero-Pascal *et al.* 2005). This type of chorionic structure is very common in eggs of other aquatic insects (Hinton 1981; Rosciszewska 1991a; Stark & Szczytko 1998), and our observations support the proposal that aeropyles are also common in eggs of Ephemeroptera.

A thin layer surrounding the whole egg, including the attachment structures, has occasionally been described in Ephemeroptera (Degrange 1960; Gaino & Bongiovanni 1993; Gaino & Mazzini 1989, 1990; Mazzini & Gaino 1985; Ubero-Pascal *et al.* 2005), although this layer is very common in other insect eggs, such as Plecoptera (Degrange 1957; Poprawa *et al.* 2002; Rosciszewska 1991a, 1991b; Stark & Szczytko 1988; Ubero-Pascal *et al.* 2001). Of the terms that have been used for this layer, we prefer to call it extra-chorion, since it defines both its radial position (the most external layer of the eggshell) and the moment of its synthesis (after of the chorion and attachment structures have been synthesized). Observation of the extra-chorion may be related to fixation or the procedures used for preparing eggs for microscope analysis (Degrange 1960; Ubero-Pascal *et al.* 2005). Therefore, our interest in the extrachorion in this paper is not so much as to describe its function (little known in Ephemeroptera), but draw attention to the fact that it should be taken into account since its presence could lead to misinterpretation of egg morphology using SEM, since it can hide the chorionic structures as has been observed in *P. luteus*.

The use of SEM as the predominant technique in egg morphology studies should not be understood as a rejection of light microscopy for this kind of study, since both techniques are necessary and compatible. In fact, SEM generally allows observation of the chorion surface in great detail, while light microscopy is necessary for morphological analysis of structures entering into the chorion or hidden by any other structure, as frequently is the case with the micropylar canal. Therefore, and in agreement with Gaino and Bongiovanni (1992), we believe that correct morphological description of eggs must be based on observations using both techniques. Besides, we also believe that a revision of the chorionic structure classification provided by Koss and Edmunds (1974) is necessary, specially as regards to attachment structures. Just as it has been possible to check *P. luteus* and *O. rhenana*, we are convinced that more changes in type designation of chorionic structures in the eggs of other species already studied by light microscope are possible.

The taxonomic usefulness of eggshell morphology in Ephemeroptera has been widely demonstrated, although care must be taken with the choice of chorionic structures for this purpose, since certain features show a high degree of intraspecific variability even among eggs of the same specimen (Degrange 1960; Gaino & Bongiovanni 1992). The size of the egg is one of these characteristics because egg dimensions can be influenced as much by biological factors (life cycles, feeding, size of female abdomen and egg number relationships, etc.) as by the way they are handled in the laboratory (fixation, preservation, procedures of egg preparation for microscope study, etc) (Brittain 1982; Degrange 1960; Soldan 1979; Ubero-Pascal *et al.* 2005). Therefore, the egg sizes indicated for the three species studied are merely illustrative and of no taxo-

onomic validity, since the range of such variations measured by us is lower than the range measured by Degrange (1960). Polar cap form is another feature that must be treated with care, because this shape has been found to vary in eggs of *P. luteus* by us and in the eggs of *S. ignita* by Gaino and Bongiovanni (1992).

Finally, the morphology patterns of the eggshell in many species of Potamanthidae, Ephemeridae and Oligoneuridae must be studied using SEM, in order to check whether the morphological features of the chorion described in this paper for *P. luteus*, *E. danica* and *O. rhenana* may be useful for taxonomic purposes. We are sure that egg features given in this paper will be very useful for systematic studies in Ephemeroptera.

## References

- Alba-Tercedor, J. (2000) *Habrophlebia antoninoi* sp. n., a new species from Spain, with an account of the European species of *Habrophlebia* Eaton, 1881 (Ephemeroptera: Leptophlebiidae, Habrophlebiinae). *Aquatic Insects*, 22, 17.
- Alba-Tercedor, J. & El Alami, M. (1999) Descriptions of the nymphs and eggs of *Acentrella almohades* sp. n. from Morocco and Southern Spain (Ephemeroptera: Baetidae). *Aquatic Insects*, 21, 241–247.
- Alba-Tercedor, J. & Sowa, R. (1987) New representatives of the *Rhithrogena diaphana*-group from Continental Europe, with a redescription of *R. diaphana* navás, 1917 (Ephemeroptera: Heptageniidae). *Aquatic Insects*, 9, 65–83.
- Balasubramanian, C., Venkataraman, K. & Sivaramakrishnan, K.G. (1991) Life stages of a South Indian burrowing mayfly, *Ephemerella (Aethephemerella) nadinae* McCafferty and Edmunds 1973 (Ephemeroptera: Ephemeridae). *Aquatic Insects*, 13, 223–228.
- Belfiore, C., Haybach, A. & Klonowska-Olejnik, M. (1999) Taxonomy and phenetic relationships of *Electrogena affinis* (Eaton, 1883) (Ephemeroptera, Heptageniidae). *Annales de Limnologie*, 35, 245–256.
- Bengtsson, S. (1913). Undersökningar öfver äggen hos Ephemeriderna. *Entomologisk Tidskrift*, 34, 271–320.
- Brittain, J.E. (1982) Biology of Mayflies. *Annual Review of Entomology* 27, 119–147.
- Degrange, C. (1956) Sur les micropyles des oeufs des Éphéméroptères. *Bulletin de la Société Entomologique de France*, 61, 147–148.
- Degrange, C. (1957) L'Oeuf et le mode d'éclosion de quelques Plécoptères. *Travaux du Laboratoire d'Hydrobiologie et de Pisciculture de l'Université de Grenoble*, 48/49, 37–49.
- Degrange, C. (1960) Recherches sur la Reproduction des Éphéméroptères. *Travaux du Laboratoire d'Hydrobiologie et de Pisciculture de l'Université de Grenoble*, 51, 71–93.
- Domínguez, E. & Cuzzo, M.G. (2002) Ephemeroptera egg chorion characters: A test of their importance in assessing phylogenetic relationships. *Journal of Morphology*, 253, 148–165.
- Gaino, E. & Bongiovanni, E. (1992) Comparative morphology of epithemata (polar chorionic structures) in the eggs of *Ephemerella ignita* (Ephemeroptera: Ephemerellidae). *Transactions of the American Microscopical Society*, 111, 255–265.
- Gaino, E. & Bongiovanni, E. (1993) Scanning electron microscopy of the eggs of *Palingenia longicauda* (Olivier) (Ephemeroptera: Palingeniidae). *International Journal of Insect Morphology & Embryology*, 22, 41–48.
- Gaino, E. & Mazzini, M. (1988) Fine structure of the chorionic projections of the egg of *Rhithrogena kimminsi* Thomas (Ephemeroptera: Heptageniidae) and their role in egg adhesion. *International Journal of Insect Morphology & Embryology*, 17, 113–120.
- Gaino, E. & Mazzini, M. (1989) Chorionic adhesive material of the egg of the mayfly *Habrophlebia eldae* (Ephemeroptera, Leptophlebiidae): morphology and synthesis. *Bolletino di Zoologia*, 56, 291–298.
- Gaino, E. & Puig, P. (1996) *Choroterpes (Choroterpes) salamannai*, a new species of mayfly from Central and South West Spain (Ephemeroptera: Leptophlebiidae). *Bolletino della Societ Entomologica Italiana*, 128, 99–104.
- Gaino, E. & Rebora, M. (2001) Synthesis and function of the fibrous layers covering the eggs of *Siphonurus lacustris* (Ephemeroptera, Siphonuridae). *Acta Zoologica Stockholm*, 82, 41–48.
- Gaino, E. & Rebora, M. (2003) Adhesiveness of the eggs of *Ecdyonurus venosus* to siliceous and calcareous substrate. In: Gaino, E. (Ed.), *Research Update on Ephemeroptera & Plecoptera*. University of Perugia, Perugia, pp: 437–443.
- Gaino, E., Belfiore, C. & Mazzini, M. (1987) Ootaxonomic investigation of the Italian species of the genus *Electrogena* (Ephemeroptera, Heptageniidae). *Bolletino di Zoologia*, 54, 169–175.
- Gaino, E., Mazzini, M. & Sartori, M. (1993) Comparative analysis of the chorionic pattern in *Habroleptoides* species (Ephemeroptera, Leptophlebiidae). *Bolletino di Zoologia*, 60, 155–162.
- Gaino, E., Mazzini, M., Degrange, C. & Sowa, R. (1989) Etude en microscopie a balayage des oeufs de quelques especes de *Rhithrogena* Eaton groupe *alpestris* (Ephemeroptera, Heptageniidae). *Vie Milieu*, 39, 219–229.
- Haybach, A. (2003) Egg-structure of German mayflies (Insecta, Ephemeroptera).- an overview. *Lauterbornia*, 47, 41–58.
- Hinton, H.E. (1981) *Biology of Insects Eggs*. Pergamon Press, Oxford, pp.

- Jacobus, L.M. & Sartori, M. (2004) Review of the genus *Hyrtanella* (Ephemeroptera: Ephemerellidae). *Zootaxa*, 785, 1–12.
- Kang, S.C. & Yang, C.T. (1994) Ephemeroidea of Taiwan (Ephemeroptera). *Chinese Journal of Entomology*, 14, 391–399.
- Klonowska-Olejnik, M. (1997) The use of egg morphology in the taxonomy of some species of the genus *Rhithrogena* (Ephemeroptera: Heptageniidae). In: Landolt, P. & Sartori, M. (Eds.), *Ephemeroptera & Plecoptera: Biology - Ecology - Systematic*. MTL, Fribourg, pp. 457–462.
- Klonowska-Olejnik, M. (2004) Redescription of *Electrogena quadrilineata* (Landa, 1969) from type material (Ephemeroptera, Heptageniidae). *Aquatic Insects*, 26, 85–95.
- Klonowska-Olejnik, M. & Jazdzewska, T. (2003) Scanning electron microscopy study of the eggs of some rare mayfly (Ephemeroptera) species: *Ametropus fragilis*, *Isonychia ignota* and *Neoephemera maxima*. In: Gaino, E. (Ed.), *Research Update on Ephemeroptera & Plecoptera*. University of Perugia, Perugia, pp. 147–462.
- Kopelke, J.P. & Müller-Liebenau, I. (1981a) Eistrukturen bei Ephemeroptera un deren Bedeutung für die Aufstellung von Artengruppen am Beispiel der europäischen Arten der Gattung *Baetis* Leach, 1815 (Insecta: Baetidae). Teil II: *rhodani*-, *vernus*- und *fuscus*-Gruppe. *Spixiana*, 4, 39–54.
- Kopelke, J.P. & Müller-Liebenau, I. (1981b) Eistrukturen bei Ephemeroptera un deren Bedeutung für die Aufstellung von Artengruppen am Beispiel der europäischen Arten der Gattung *Baetis* Leach, 1815 (Insecta: Baetidae). Teil III: *buceratus*-, *atrebatinus*-, *niger*-, *gracilis*- und *muticus*-Gruppe. *Deutsche Entomologische Zeitschrift*, 28, 13.
- Kopelke, J.P. & Müller-Liebenau, I. (1982) Eistrukturen bei Ephemeroptera un deren Bedeutung für die Aufstellung von Artengruppen am Beispiel der europäischen Arten der Gattung *Baetis* Leach, 1815 (Insecta: Baetidae). Teil I: *alpinus*-, *lutheri*- und *lapponicus*-Gruppe. *Gewässer und Abwasser*, 68/69, 7–25.
- Koss, R.W. (1968) Morphology and taxonomic use of Ephemeroptera eggs. *Annals of the Entomological Society of America*, 61, 696–721.
- Koss, R.W. & Edmunds, G.F.Jr. (1974) Ephemeroptera eggs and their contribution to phylogenetic studies of the order. *Zoological Journal of the Linnean Society*, 55, 267–349.
- Malzacher, P. (1982) Eistrukturen europäischer Caenidae (Insecta: Ephemeroptera). *Stuttgarter Beiträge zur Naturkunde, A, Biologie*, 356, 1–15.
- Mazzini, M. & Gaino, E. (1985) Fine structure of the egg shell of *Habrophlebia fusca* (Curtis) y *H. Consiglioi* Biancheri (Ephemeroptera: Leptophlebiidae). *International Journal of Insect Morphology & Embriology*, 4, 327–334.
- Mazzini, M. & Gaino, M. (1990) Oogenesis and involvement of chorionic structures in Ephemeroptera taxonomy. In: Campbell, I.C. (Ed.), *Mayflies and Stoneflies*. Kluwer Academic Publishers, New York, pp. 95–104.
- Pescador, M.L. & Edmunds, G.F.Jr. (1994) New genus of Oligoneuriidae (Ephemeroptera) from South America. *Annals of the Entomological Society of America*, 87, 263–269.
- Poprawa, I., Baran, A. & Rosciszewska, E. (2002) Structure of ovaries and formation of egg envelopes in the stonefly, *Leuctra autumnalis* Aubert, 1948 (Plecoptera: Leuctridae). Ultrastructural studies. *Folia Biologica Krakow*, 50, 29–38.
- Puig, M.A. & Gaino, E. (1996) *Choroterpes* (*Choroterpes*) *pratti* n. sp., a new species of mayfly from North-East of Spain (Ephemeroptera: Leptophlebiidae). *Annales de Limnologie*, 32, 229–233.
- Rosciszewska, E. (1991a) Ultrastructural and histochemical studies of the egg capsules of *Perla marginata* (Panzer, 1799) and *Dinocras cephalotes* (Curtis, 1827) (Plecoptera: Perlidae). *International Journal of Insect Morphology & Embriology*, 20, 189–203.
- Rosciszewska, E. (1991b) Morphological changes developing after oviposition on egg capsule surface of *Isoperla rivulorum* Gurin (Plecoptera: Perlodidae). *Zoologische Jahrbcher; Anatomie*, 121, 253–258.
- Smith, O.R. (1935) The egg and eggs-laying habits of North American mayflies. In: Needham, J.G., Traver, J.R. & Hsu, Y.C. (Eds.), *The Biology of Mayflies*. Ithaca, New York, pp. 67–89.
- Soldan, T. (1979) The structure and development of the female internal reproductive system in six european species of Ephemeroptera. *Acta entomologica bohemoslovaca*, 76, 353–365.
- Soldan, T. & Thomas, A.G.B. (1983) New and little-known species of mayflies (Ephemeroptera) from Algeria. *Acta entomologica bohemoslovaca*, 80, 356–376.
- Stark, B.P. & Szczytko, S.W. (1988) Morphology and phylogeny in Arcynopterygini (Plecoptera: Perlodidae). *Journal of the Kansas Entomological Society*, 61, 143–160.
- Studemann, D. & Landolt, P. (1997) Eggs of Ephemerellidae (Ephemeroptera). In: Landolt, P. & Sartori, M. (Eds.), *Ephemeroptera & Plecoptera: Biology - Ecology - Systematic*. MTL, Fribourg, pp. 362–371.
- Studemann, D., Landolt, P. & Tomka, I. (1995) Eggs of the european Ephemerellidae (Ephemeroptera). In: Corkum, L.D. & Ciborowski, J.H. (Eds.), *Current direction in research on Ephemeroptera*. Canadian Scholars' Press Inc, Toronto, pp. 407–422.
- Thomas, A.G.B., Gaino, E. & Maire, V. (1999). Complementary descripton of *Habrophlebia vaillantorum* Thomas, 1986 in comparison with *H. fusca* (Curtis, 1843) (Ephemeroptera, Leptophlebiidae). *Ephemera*, 1, 9–21.
- Towns, D.R. & Peters, W.L. (1979) New genera and species of Leptophlebiidae (Ephemeroptera) from New Zealand.



*New Zealand Journal of Zoology*, 6, 439–452.

- Ubero-Pascal, N. (2004) *Estudio Morfológico del huevo en los Órdenes Ephemeroptera y Plecóptera. Aplicación Taxonómica en la cuenca del Río Segura (S.E. de la Península Ibérica)*. Unpublished D. Phil. Thesis, Murcia University, Murcia, 580 pp.
- Ubero-Pascal, N., Puig, M.A. & Soler, A. (1998) Los Efemerópteros de la cuenca del río Segura (S.E. de España): 1. Estudio Faunístico (Insecta: Ephemeroptera). *Boletín de la Asociación Española de Entomología*, 22, 151–170.
- Ubero-Pascal, N., Soler, A. & Puig, M.A. (2001) Contribution to the egg morphology of the spanish stoneflies (Plecoptera). An overview in the Segura river basin (SE of Spain). In: Domínguez, E. (Ed.), *Trends in Research in Ephemeroptera and Plecoptera*. Kluwer Academic/Plenum Publishers, New York, pp. 467–478.
- Ubero-Pascal, N., Fortuño, J.M. & Puig, M.A. (2005) New application of air-drying techniques for studying Ephemeroptera and Plecoptera eggs by Scanning Electron Microscopy. *Microscopy Research and Technique*, 68, 264–271.

